

## LETTER TO JMG

## Genetic susceptibility in familial melanoma from northeastern Italy

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Cutaneous malignant melanoma (CMM) is a potentially fatal form of skin cancer, whose incidence and mortality are increasing in the Western world.<sup>1,2</sup> Approximately 3–12% of cutaneous malignant melanoma develops in families with multiple cases of melanoma.<sup>3–5</sup> Worldwide studies of large families prone to melanoma have demonstrated linkage to a locus on chromosome 9p21 (MIM 600160) in the majority of kindreds, and probable linkage to 1p22<sup>6</sup> and 1p36<sup>7,8</sup> in others. About one third of 9p21 linked families carry mutations in the *CDKN2A* tumour suppressor gene,<sup>9,10</sup> which encodes the p16 cell cycle inhibitor. Rare kindreds may also possess mutations of the coding sequences of *CDK4* (MIM 12829),<sup>11,12</sup> or *p14<sup>ARF</sup>* (translated from exons 1β and 2 of *CDKN2A*).<sup>13–15</sup> More studies are needed to understand the genetic basis of melanoma.

In Italy, the melanoma prone families studied to date are mostly from the north<sup>16</sup> and northwestern areas,<sup>15,17,18</sup> the centre,<sup>19</sup> and the Sardinia region.<sup>20</sup> These families are generally characterised by small numbers of melanoma cases, and a relatively high frequency of *CDKN2A* mutations.

We studied families prone to melanoma from northeastern Italy, to characterise genetic susceptibility to melanoma in this population.

## MATERIALS AND METHODS

## Study population

The study group was comprised of 55 families: 44 (80%) from southern Emilia Romagna and northern Marche (close to the border between the two regions); 10 families, all with two cases per family, from other areas of Italy (four from the south, three from the centre, two from the north, and one from Sardinia), and one family from Russia. All the families were recruited at the Dermatology Unit of the Bufalini Hospital in Cesena, Italy. Bufalini Hospital's and the National Cancer Institute's Ethical Committees approved the study, and written informed consent was obtained from all participants. In the study period, the Bufalini Hospital examined approximately 85% of all cutaneous malignant melanoma patients from the Marche and Emilia Romagna area, as verified with the Romagna regional cancer registry<sup>21</sup> and with records of melanoma diagnoses from the main hospitals of the area.<sup>22</sup> Thus, the families coming from Marche and Emilia Romagna region are representative of the study area. The characteristics of these families are reported in table 1. All subjects answered an interview based questionnaire and donated a blood sample, which was immediately processed to obtain cryopreserved lymphocytes and serum samples. A single dermatologist (DC) examined all subjects, and diagnosed melanoma and dysplastic nevi. A dysplastic nevus had to be  $\geq 5$  mm, predominantly flat, and have at least two of the following criteria: variable pigmentation, indistinct borders, and irregular outline.<sup>23</sup> All cases of melanoma and any other tumours in the cases of melanoma were confirmed histologically. Melanoma

## Key points

- We studied 55 families prone to melanoma and having at least two relatives with melanoma, mostly from the Emilia Romagna and Marche regions of Italy. Cases with multiple primary melanomas, dysplastic nevi, and pancreatic cancer were present in several families. We sequenced *CDKN2A* in all families, and screened *CDKN2B*, *p14<sup>ARF</sup>*, and *CDK4* in a subset. In families with more than one case of breast or gastric cancer, we also sequenced *BRCA2* and *CDH1*. We performed linkage analysis of chromosomes 1 and 9, which harbour loci previously linked to melanoma susceptibility. We examined the effect of a novel *CDKN2A* mutation on the p16 protein tertiary structure and function.
- Three known p16 mutations, G101W, R24P, and S56I, and a novel L65P were identified in four kindreds, accounting for 7% of all families. The L65P mutation caused a small distortion of p16 tertiary structure and reduced binding to CDK4 by 50% in a yeast two hybrid assay. No disease related mutations were found in the other tested genes. There was no evidence for linkage to either chromosome 1 or 9.
- We conclude that germline alterations of one or more genes other than *CDKN2A*, and on chromosome loci other than those previously identified, may be important for melanoma predisposition. The population of northeastern Italy may be a rich source for further genetic studies on melanoma.

diagnosis was reviewed in 40% of the cases by three independent pathologists, with 99% concordance with the original diagnosis.

## CDKN2A gene analysis

The promoter and the three exons of *CDKN2A* were amplified from the genomic DNA extracted from the patient blood samples. The reaction mix for PCR amplification of the promoter region included 1×PCR buffer (Invitrogen High Fidelity PCR buffer); 2.2 mM MgSO<sub>4</sub>; 5% DMSO; 175 nM of each pair of primers; 50 nM of each of the four dNTPs and 1 unit of HiFi Platinum Taq polymerase (Invitrogen). This mix was also used in the amplification of the three exons with changes of 2 mM MgSO<sub>4</sub> and 5% DMSO for exons 1 and 2, and 3 mM MgSO<sub>4</sub> and 3% DMSO for exon 3 in the final PCR mix. The PCR for the promoter and exon 3 were

**Abbreviations:** BCC, basal cell carcinoma; CMM, cutaneous malignant melanoma; DN, dysplastic nevi; STS, soft tissue sarcoma

programmed with 40 cycles of amplification on a Perkin Elmer PCR cycler. The amplification programme of each cycle consisted of 60 s at 95°C, 60 s at the annealing temperature and 60 s at 72°C. A 3 min initial denaturing step at 95°C and a 10 min final extension step at 72°C were included in both PCRs. The annealing temperature was 55°C for the amplification of the promoter and 58°C in exon 3. The PCR of exons 1 and 2 involved a "touchdown" thermal cycling with two cycles at each decreased annealing temperature. The method then followed that of Castellano et al.<sup>24</sup> and Pollock et al.<sup>25</sup> All products were run on DNA 1000 labchips using "lab-on-a-chip" capillary electrophoresis (Agilent, Palo Alto, CA). All PCR products were treated with shrimp alkaline phosphatase and exonucleaseI (ExoI),<sup>26</sup> and followed by the use of a 96 well plate of G-50 Sephadex to clean the PCR product before sequencing. All products were sequenced with ABI prism BigDye terminator cycle sequencing kit 1.0 (Applied Biosystems Inc.) on an ABI3700 sequence analyser. The PCR primers of the promoter and exon 2 were previously described by Pollock et al.<sup>25</sup> The DNA sequences of the primers used for *CDKN2A* amplification and sequencing were: promoter, amplification: P96F and P968R,<sup>25</sup> sequencing: 2F-TGCCCCAGACAGCCGTTTAC; 1R-ACGACTAGAAAGTGTCC; with P96F and P968R; exon 1, amplification: P781F<sup>25</sup>; 4R-GCGCTACCTGATTCCAATTC; sequencing: 4F-CAGCACCG GAGGAAGAAAG; 3R-TGCCTGCTCTCCCTCT; with P781F and 4R; exon 2, amplification with 42F and 551R,<sup>25</sup> sequencing with 5'-CTGAACCTTTCTGTGCTGG and 42F;<sup>25</sup> exon 3, amplification, and sequencing with 11F-CAGTGGA CTAGCTGCTGGAC; 11R-ATGGACATTTACGGTAGTGGG. All sequences were analysed and variants were detected using Mutation Surveyor (SoftGenetics Inc., PA).

#### Analysis of *P14<sup>ARF</sup>* ( $\beta$ transcript), *CDKN2B*, and *CDK4* genes

PCR was performed by amplifying 30 ng of genomic DNA in 20  $\mu$ l in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, 6 pmol of each primer, and 0.02 units of Taq Gold DNA polymerase (Perkin-Elmer). Cycling conditions included an initial 94°C denaturation step for 10 min, then 35 cycles of 94°C denaturation for 30 s, 57–62°C annealing for 1 min, and a 72°C extension step for 1 min, ending with a 72°C step for 5 min.

PCR for SSCP was conducted with the addition of 0.04  $\mu$ l of a32P-dCTP (3000Ci/mMol, New England Nuclear Inc.) to each reaction. Following PCR, 10  $\mu$ l of stop solution (0.05% each of bromphenol blue and xylene cyanol in 100% formamide) were added to each reaction. The products were heated at 95°C for 5 min and then quickly chilled on ice. Products were separated in vertical acrylamide slab gels.

PCR products were directly sequenced using Big Dye chemistry (Applied Biosystems Inc. (ABI). Products were

purified using Qiagen Inc. quick spin columns. Electrophoresis was carried out on an ABI 377 automated fluorescent DNA sequencing machine. Nucleotide sequences were aligned and analysed using Sequencer software (Gene Codes Inc.). Primers for *CDKN2B* were as described in Liu et al.<sup>27</sup>; primers for the  $\beta$  transcript were as described in Mao et al.<sup>28</sup> and primers for *CDK4* were described in Zuo et al.<sup>29</sup> and Shennan et al.<sup>30</sup>

#### Genome wide scan of chromosomes 1 and 9

The marker set genotyped in this study was the Applied Biosystems Inc. (ABI) linkage mapping set version 2.5 (MD-10) with 31 (for chromosome 1) and 20 (for chromosome 9) dinucleotide microsatellites arranged into panels with an average heterozygosity of 0.79 and a resolution of approximately 10 cM between markers. Microsatellites were genotyped by consolidating the amplification reactions into 3–5 reactions per panel, each containing 2–5 markers. The products of each multiplex were pooled by panel, diluted and mixed with deionised formamide and size standard before electrophoresis. Multiplex PCRs were designed with respect to dye colour, marker size, and quality of amplification product obtained. Failed amplifications were re-amplified either singly or in redesigned multiplexes.

Each PCR reaction mix contained primers of 0.15–5 pmol per marker, 20 ng of genomic DNA, and 3  $\mu$ l of ABI True Allele Mix in a total reaction volume of 5  $\mu$ l. Reaction conditions (MJ Research PTC-225 Tetrad) were 95°C for 12 min followed by 10 cycles at 94°C for 15 s, 53°C for 15 s, and 72°C for 30 s and then 22 cycles at 89°C for 15 s, 53°C for 15 s, and 72°C for 30 s with a final 10 min extension step at 72°C. Following amplification each multiplex was diluted to 10  $\mu$ l with ddH<sub>2</sub>O and 2  $\mu$ l were pooled per individual ABI panel. Two microlitres of each pooled panel or individual multiplex were mixed with 0.4  $\mu$ l GS500LIZ size standard and 7.6  $\mu$ l deionised formamide prior to electrophoresis. Electrophoresis was performed in either 96 or 384 well plates on an ABI 3100 capillary electrophoresis instrument. Fluorescent peak data were sized using ABI GeneScan Analysis version 3.7 and allele calling performed using Genotyper 3.7 software. Allele calling consistency between plates was verified through positive controls and verification of individual genotypes obtained by concordance of the pooled multiplex genotypes with those of the individual multiplexes. Genotypes were assigned blind to pedigree structure and clinical data.

#### Linkage analysis of chromosomes 1 and 9

Power calculations based on simulation studies on the 47 families tested for linkage revealed 85% power to detect a lod score higher than 3 (expected maximum lod score of 4.15), under a dominant model with 50% penetrance and the

**Table 1** Family characteristics

No CMM in family	No families	Mean age at CMM diagnosis*	Families with			Other cancers in melanoma cases	No families by region
			MPM	DN	PC		
2	42	50	11	40	2	8 subjects: adnexal, BCC, breast, kidney, STS, testicular, thyroid throat 2 subjects: BCC, kidney, prostate	32=Emilia Romagna+Marche; 10=others 25=Emilia Romagna+Marche; 9=Abruzzi, Campania, Friuli, Puglia, Sardinia, Tuscany
1 <sup>st</sup> degree	34	51	6	33	2		
$\geq 2^{\text{nd}}$ degree	8	46	5	7	0		
3	11	48	3	9	0		
4	1	48	1	1	1		
5	1	43	0	1	0	0	1=Emilia Romagna 1=Emilia Romagna

CMM, cutaneous malignant melanoma; MPM, multiple primary melanomas; DN, dysplastic nevi; PC; pancreatic cancer, BCC; basal cell carcinoma; STS, soft tissue sarcoma

\*For multiple primary melanomas, the first diagnosed melanoma was considered

assumption of no heterogeneity, using a simulated marker with six equally frequent alleles.

Parametric two point and multipoint linkage analyses for chromosomes 1 and 9 were performed, using MLINK (v. 5.1) of the FASTLINK (v. 4.1p) software package,<sup>31,32</sup> and GENEHUNTER (V. 2.0β),<sup>33</sup> respectively, assuming an autosomal dominant inheritance model with 50% penetrance. The disease frequency under the dominant model was calculated as 0.0004 based on the prevalent sex averaged rate of melanoma in the Italian population of 60/100 000.<sup>34</sup> Only individuals diagnosed with cutaneous malignant melanoma were coded as affected. For genotyped data, Mendelian inconsistencies were found in 0.15% using PEDCHECK (v. 1.1).<sup>35</sup> Erroneous genotypes were reread, changed, or dropped because of non-paternity (0.04%). Marker allele frequencies were calculated using RECODE (v. 1.4) (<http://watson.hgen.pitt.edu/register>), based on founder and married-in individuals. The analysis was conducted on the entire set of families after the exclusion of non-informative families or families with positive mutations in candidate genes: 47 families were used for the analysis of chromosome 1 and 46 families for chromosome 9. We also repeated the analysis excluding the eight families with melanoma only in second or further degree relatives. The exclusion did not appreciably alter the results. Thus, data from these eight families are reported in the manuscript.

Non-parametric multipoint linkage analysis was also performed using GENEHUNTER (v. 2.0 β). The Marshfield genetic maps<sup>36</sup> were used to determine order and distance between markers. We tested for genetic heterogeneity using GENEHUNTER (v. 2.0 β) and HOMOG (v. 3.35).<sup>37,38</sup>

### Analysis of the 3D structure of the p16 INK4a protein

All predictive mutational effects on protein structure were carried out using publicly available web services, as reported in Guerois et al<sup>39</sup> for FOLD-X calculations; in Gilis et al<sup>40</sup> for PoPMuSiC calculations; and in Sunyaev et al<sup>41</sup> for PolyPhen calculations.

### Analysis of the interaction of p16 wild-type and mutant to CDK4 by a yeast two hybrid system

The growth and maintenance of *S. cerevisiae* was performed using methods described in Guthrie and Fink.<sup>42</sup> Two hybrid analysis was carried out in the yeast strain RAY43 (MATa *ura3-52 his3-200 leu2-3, -112; trp1-901; Can r gal4-542; gal80-538; ade2::GAL1-URA3; lys2::lexA<sub>opx4</sub>-lacZ; met3::lexA<sub>opx4</sub>-HIS3*). Both pBTM116<sup>43</sup> and pACTII (Clontech) were used for two hybrid analysis. The strategies used to generate the vectors pBTM116-p16WTlexA, pBTM116- p16L65PlexA, and pACTII-GAL4ADCDK4 are available on request. All two hybrid vectors were sequenced to confirm the absence of PCR generated nucleotide substitutions and to ensure that the appropriate frame was maintained throughout the entire fusion gene. Plasmids were transformed into the RAY43 strain as described in Gietz and Woods.<sup>44</sup> Quantitative analysis of β-galactosidase activity was performed using a modification of the method of Miller.<sup>45</sup> Mean values for liquid β-galactosidase assays were derived from 28 independent experiments at 30°C and 20 separate experiments at 37°C. The β-galactosidase activity (defined in Miller units) was measured using the following formula:

$$\beta - \text{galactosidase activity} = \frac{(1000 \times OD_{570})}{(\text{Time [min]} \times \text{Volume [ml]} \times OD_{405})}$$

p16WT and p16L65P β-galactosidase activity were compared assuming homoscedasticity using a two tailed Student's *t* test.

### CDH1 gene analysis

PCR was performed by amplifying 2.0 μl of genomic DNA in a 25 μl reaction containing 2.5 μl of 10×buffer, 0.2 μl of dNTPs, 0.1 μl of Taq Gold DNA polymerase (Perkin-Elmer), 2.0 μl of oligonucleotide primers,<sup>46</sup> and 18.2 μl of water. Cycling conditions included an initial 94°C denaturation step for 10 min, followed by 12 cycles of 94°C denaturation for 12 s, 65°C annealing for 20 s, and a 72°C extension step for 55 s with a reduction of 0.5°C of annealing temperature with each cycle. The procedures then followed 30 cycles: 94°C denaturation for 12 s, 50°C annealing for 20 s, and a 72°C extension step for 55 s, ending with a 72°C step for 7 min.

PCR products were directly sequenced using the High Pure Plasmid Isolation Kit (Boehringer Mannheim, Inc.). Electrophoresis was carried out on an ABI 310 automated DNA sequencing machine. Nucleotide sequences were aligned and analysed using Seqman software.

### BRCA2 gene analysis

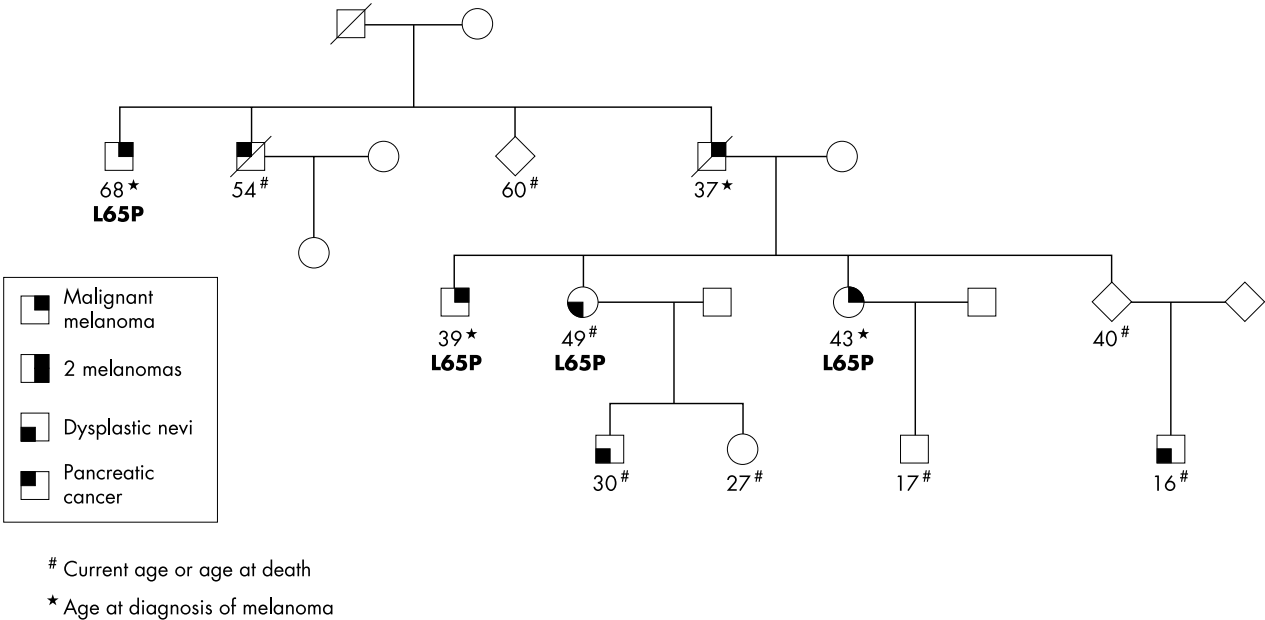
Full sequence determination in both forward and reverse directions of approximately 10 200 base pairs containing 26 coding exons and approximately 900 adjacent base pairs in the introns (not more than 20 base pairs proximal to the 5' end and 10 base pairs distal to 3' end of each exon) was conducted under contract at Myriad Genetics Laboratories. Exon 1, which is non-coding, was not analysed. Patient DNA was subjected to 47 PCR reactions. The amplified products were each directly sequenced in forward and reverse directions using fluorescent dye labelled sequencing primers. Chromatographic tracings of each amplicon were analysed by a proprietary (BRACAnalysis, Myriad Genetics Laboratories) computer based review followed by visual inspection and confirmation.

## RESULTS

### CDKN2A gene

We sequenced *CDKN2A* in melanoma affected and unaffected members of 55 families. Four mutations in the *CDKN2A* gene were found in seven different members of four kindreds (table 2). Three mutations, G101W, R24P, and S56I, have already been found in many families, including Italians.<sup>16,19,47</sup> The fourth potentially disease related mutation we found was a novel T to C transition at bp 194 of exon 2, which results in a missense mutation L65P in p16 and a silent mutation A79A in p14<sup>ARF</sup>. This mutation was found in a family with one patient having pancreatic cancer and four patients with melanoma, one of whom had two primary melanomas (fig 1). Three cases of cutaneous malignant melanoma and one unaffected family member, who had had several nevi removed for suspected but not confirmed melanoma, carried this mutation. The fourth patient with melanoma was deceased and could not be tested for this mutation. We also found the same mutation in a 39 year old unaffected member of another family from the same geographic area with five individuals with melanoma. Unfortunately, all of these individuals except one were deceased, and thus could not be tested for any *CDKN2A* mutation. The only living individual with cutaneous malignant melanoma, and seven other unaffected family members, did not carry *CDKN2A* mutations.

Among the families positive for mutations, 75% of carriers were affected. In these subjects, melanoma lesions were either of the superficial spreading or nodular histologic type, with thicknesses ranging from 0.8 mm to 10 mm, and Clark levels from II to IV. Their age at diagnosis did not differ significantly from that of the cases with no detected *CDKN2A* mutations. All the mutations were found in families from the Emilia Romagna region, with the exclusion of R24P, which was found in a family from Naples (Campania).



**Figure 1** Pedigree of the family with p16 L65P mutation, from the Emilia Romagna region, Italy.

We found two additional alterations that do not appear to be related to disease in two other families with two first degree relatives with melanoma: a mutation of intron 1 (IVS1+38), present only in a 12 year old unaffected individual, and a G to A mutation at bp 384 in exon 2, which results in a silent mutation R128R (and occurs on the 3' UTR of *p14<sup>ARF</sup>*), present in one affected and one 54 year old unaffected individual from the same family.

We also found several known polymorphisms and a sequence variant of uncertain significance in *CDKN2A* exon 2, exon 3, and the promoter area. Specifically, 10 families carried the exon 2 polymorphism at bp 442, which results in the A148T amino acid change.<sup>48</sup> Five sequence variants were found in the promoter area: P-493<sup>48</sup> was found in nine of the ten subjects who carried the A148T variant, consistent with the previously reported linkage disequilibrium between these two loci<sup>47, 48</sup>; P-981<sup>25</sup> was found in five families; P-735<sup>48</sup> was found in two families; P-191<sup>48</sup> was found in 59% of the patients and in 74% of the unaffected individuals; and a novel P-745 was found in one patient only. This cutaneous malignant melanoma patient also carried the A148T and P-493 variants, but had no mutations in the coding region. In the 3' UTR, the 500 G>C polymorphism<sup>49</sup> was found in 28%

of cases and 37% of unaffected individuals; the 540 C>T polymorphism<sup>49</sup> was found in 23% of cases and 24% of unaffected individuals.

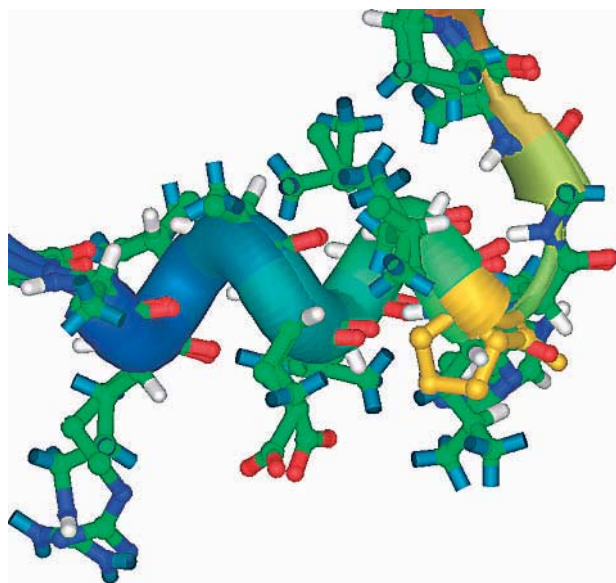
We analysed the changes in the tertiary structure of the p16 protein due to the novel L65P mutation. Leu65Pro (fig 2) is at the last residue position in helix 6 and thus could shorten the helix and disrupt secondary structure. It is unclear how this would alter the tertiary structure, but the solvent accessibility at this position would suggest a relatively small distortion. The removal of the methyl groups at this position could disrupt external interactions with other proteins. Both the FOLD-X calculations (ddG = 21.38 kJ/mol), and the PoPMuSiC calculations (ddG = 5.77 kJ/mol) revealed a difference in folding energy between the mutant and wild-type protein, but the PolyPhen predictions, based on alignment, failed to predict this mutation to be disruptive on protein function (the leucine at position 65 is not highly conserved in mammals).

To confirm the conclusions derived from protein modeling, we elected to test the interaction of the L65P mutant to CDK4 using a yeast two hybrid system. We expressed the mutant and wild-type cDNA sequences in the presence or absence of CDK4 and measured their corresponding

Table 2 Characteristics of families with <i>CDKN2A</i> mutations									
<i>CDKN2A</i> mutations		No CMM in family	Breslow and Clark levels <sup>†</sup>	Mean age at diagnosis in cases with mutations <sup>*</sup>	MPM	DN	Pancreatic cancer in family	Other cancers in family	Region
p16	p14ARF								
G101W	R115L	2	B = 10 mm C = IV	59	No	No	Yes	gastric, prostate, uterine bladder	Emilia Romagna
R24P	–	2	B = 1 mm C = III	22	No	Yes	No	breast	Campania
S56I	Q70H	2	B = 1.3 mm C = IV	61	Yes	Yes	No	breast, lung, prostate	Emilia Romagna
L65P	A79A	4	B = 0.8–2.8 mm C = II–IV	47	Yes	Yes	Yes		Emilia Romagna

CMC, cutaneous malignant melanoma; MPM, multiple primary melanomas; DN, dysplastic nevi.  
<sup>\*</sup>For multiple primary melanomas, the first diagnosed melanoma was considered.  
<sup>†</sup>Melanoma thickness in cases with *CDKN2A* mutations. The fourth family had three cases with L65P. The range of melanoma thickness in the three cases is reported.  
<sup>‡</sup>In first degree relatives.





**Figure 2** Structural model of the leucine to proline substitution at position 65 of p16 protein. The model shows that the proline amino acid (in bright yellow), unlike the leucine (behind the proline, in green), no longer makes hydrogen atoms available to the surface of the protein, possibly affecting the ability of this protein to complex or bind with its ligand.

interactions using a liquid  $\beta$ -galactosidase assay. In repeated experiments, we observed a significant decrease of  $\approx 50\%$  in the binding of L65P with CDK4, compared with the wild-type p16 at both  $30^\circ$  ( $p = 0.002$ ) and  $37^\circ$  ( $p = 0.009$ ; Student's  $t$  test) (fig 3). These results are consistent with the predictions of the protein modelling, in that the effect of the L65P mutation on CDK4 binding is rather modest. In contrast to other p16 missense mutations,<sup>50</sup> the L65P mutant does not demonstrate any temperature dependence on binding of CDK4.

#### ***P14<sup>ARF</sup>* ( $\beta$ transcript), *CDKN2B*, and *CDK4* genes**

Among the 36 families tested for *CDK4* mutations, one family had a *CDK4* alteration in exon 7: a G to A transition at 3170 bp, which changed the amino acid 240, arginine to glutamine. The proband with multiple melanomas, his unaffected mother (83 years old) and one of his unaffected sons (23 years old) showed this same mutation. However, the other cutaneous malignant melanoma case in this family did not have this mutation, so the importance of this alteration is unclear. No other disease related mutations were found in *CDK4*.

We tested 23 families for germline mutations in *CDKN2B* or *p14<sup>ARF</sup>* (exon  $\beta$ ) but detected no mutations in the coding region of either gene. Cases from two families had the *CDKN2B* polymorphism rs2069426 in intron 1 (IVS1-27). Genotype frequencies of this single nucleotide polymorphism were measured using an MboI RFLP in 65 European Americans and 89 African-Americans.<sup>51</sup> The heterozygote frequency was 20.0% and 10.1%, and the homozygote frequency was 1.5% and 2.2% in the two populations, respectively.

#### ***CDH1* and *BRCA2* genes**

In addition to the three families with pancreatic cancer, additional tumours have been reported in the families, but not histologically confirmed. In 19 families with one or more relatives with gastric cancer, and in three families with multiple breast cancer cases, in addition to the melanoma

cases, we sequenced the *CDH1* and *BRCA2* genes, respectively. Mutations or altered expression of the *CDH1* gene (MIM \*192090), on chromosome 16q22.1, have been associated with inherited diffuse gastric cancer.<sup>46-52</sup> *BRCA2* gene (MIM \*600185), on chromosome 13q12.3, is a known susceptibility gene for breast cancer<sup>53</sup> and melanoma.<sup>54</sup> In our study, we failed to detect any mutations in the coding region of either the *CDH1* or *BRCA2* genes. The families screened for *CDH1* and *BRCA2* were also negative for mutations in the other melanoma candidate genes tested.

#### **Linkage analysis of chromosomes 1 and 9**

After the exclusion of non-informative families and those with disease related gene mutations, 47 and 46 families were used for linkage analysis of chromosome 1 and 9, respectively.

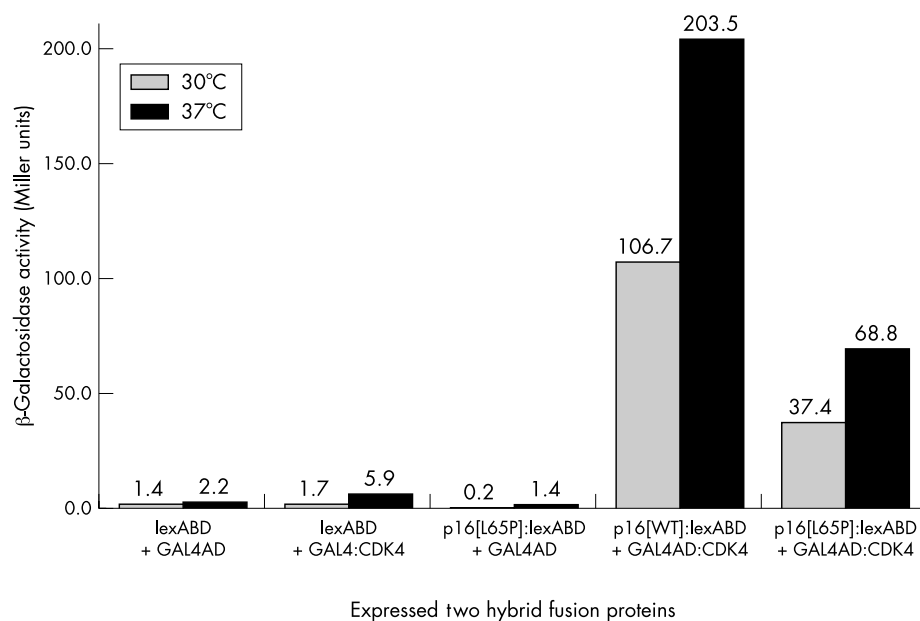
We performed two point and multipoint linkage analyses under a dominant model with 50% penetrance, consistent with the inheritance pattern for *CDKN2A* in European families,<sup>15</sup> and similar to the 1p linkage studies.<sup>6-8</sup> There was no evidence for linkage to either chromosome 1 or 9 (figs 4 and 5) in both parametric and non-parametric analyses. There was no significant evidence for genetic heterogeneity. For chromosome 1, the maximum two point lod score was 0.33 ( $= 0.25$ ) at D1S207 (p22.3). For chromosome 9, the maximum two point lod score was 0.67 ( $= 0.15$ ) at D9S161. The markers flanking *CDKN2A* (D9S157 and D9S171) had negative lod scores.

#### **DISCUSSION**

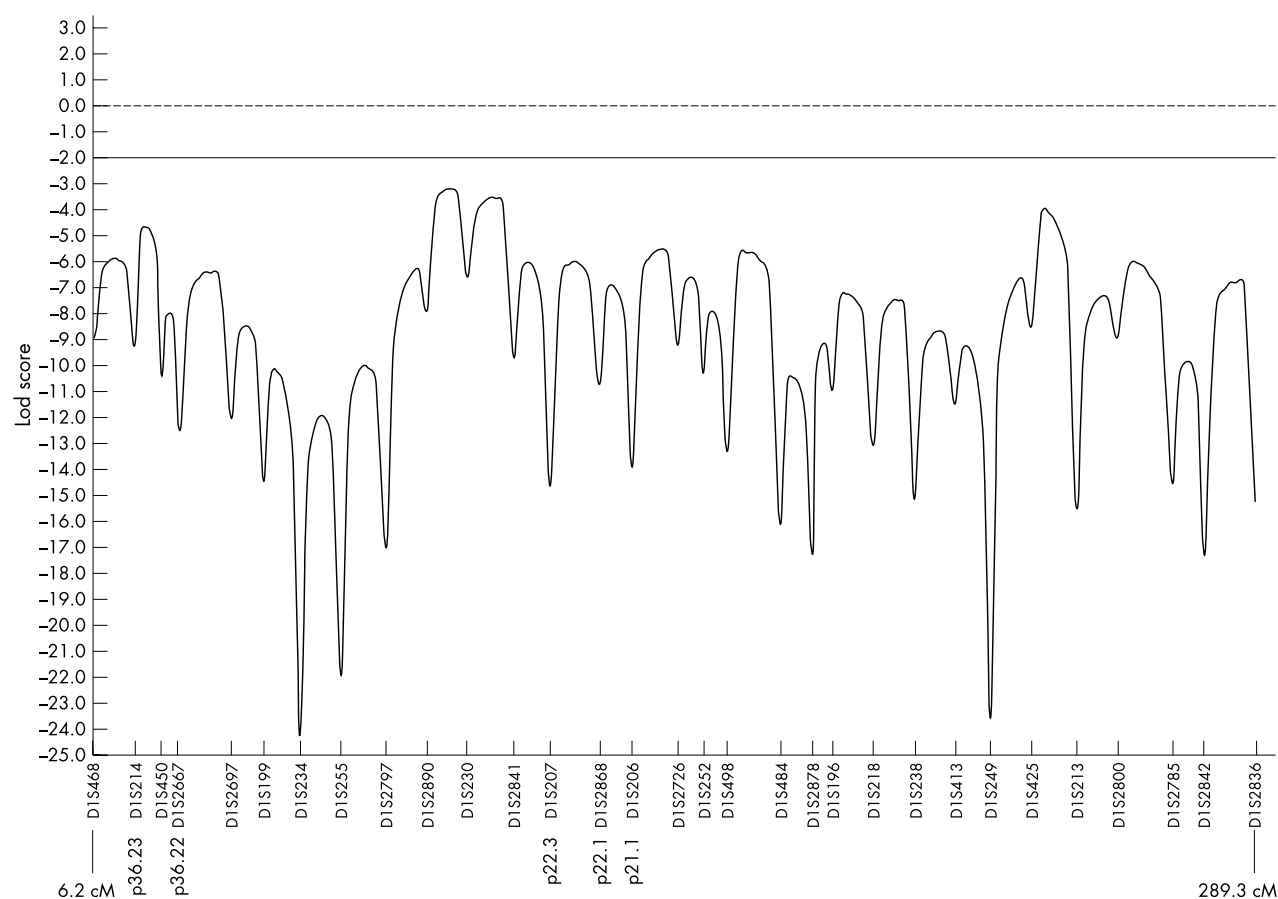
We studied a large number of Italian families, mostly from the Emilia Romagna and Marche regions, and found a low frequency of *CDKN2A* mutations, no mutations in other known candidate genes, and no evidence for linkage with previously suggested loci for possible melanoma susceptibility genes.

Melanoma aetiology is complex, involving both heterogeneous genetic and environmental components.<sup>55</sup> The known susceptibility genes explain only approximately 25% of familial melanoma worldwide, and the frequency of *CDKN2A* mutations in familial melanoma varies according to the number of cases in the families, the presence of multiple melanomas in the same patient,<sup>56-57</sup> the history of pancreatic cancer cases in the family,<sup>58</sup> and their geographical location.<sup>15-59-62</sup> Even the average lifetime risk conferred by *CDKN2A* mutations shows significant variation between regions, with the lowest penetrance in the south European countries.<sup>15</sup> Moreover, subjects in families prone to melanoma often carry multiple common melanocytic nevi and atypical or dysplastic nevi,<sup>63</sup> but development of melanoma through pathways that do not include nevi formation are likely, and they may be linked to different genes.<sup>64</sup> Indeed, a few families in our study group, also including the kindred with the G101W mutation and a case of pancreatic cancer, had no appreciable dysplastic nevi.

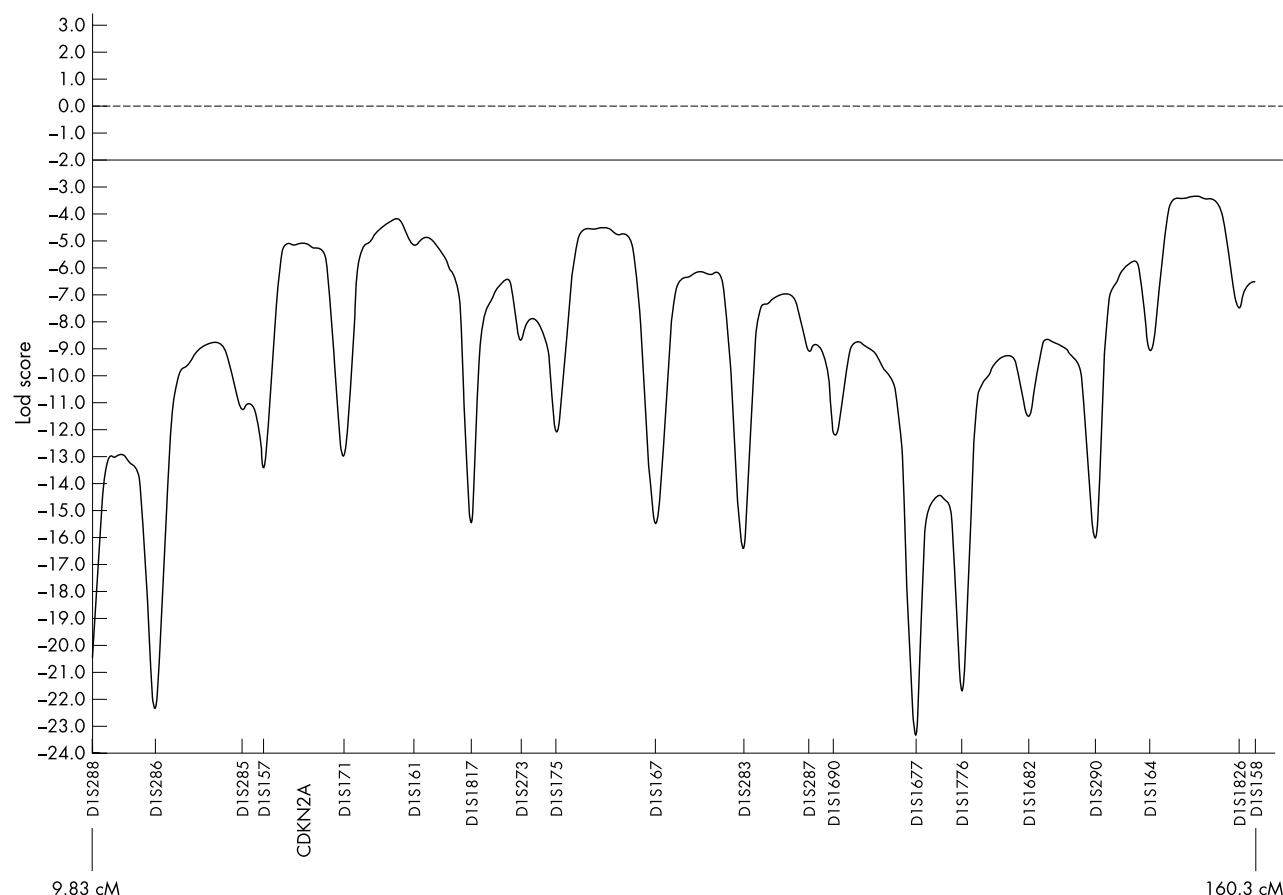
Previous studies conducted in Italian melanoma prone families have shown a relatively high proportion of *CDKN2A* mutations in the kindreds. In a study conducted in families coming mostly from the Liguria region—that is, in the northwestern part of Italy, where a founder mutation in exon 2, G101W, has been identified,<sup>65</sup> the frequency of mutations ranged from 7% in families with only two cases, to 67% in families with three cases, and 100% in families with four cases.<sup>18-47-65</sup> In central Italy, 4 out of 10 melanoma kindreds with at least two cases in first or second degree relatives had *CDKN2A* mutations (40%)<sup>19</sup> while in the Milan area, in northern Italy, 25% of families with two cases and 50% of families with three cases had *CDKN2A* mutations.<sup>16</sup> In our study, *CDKN2A* mutations were present in 7.3% of melanoma



**Figure 3** Abnormal p16 (L65P) binding to CDK4 in the yeast two hybrid system. Quantitative analysis of  $\beta$ -galactosidase activity was performed using a liquid  $\beta$ -galactosidase assay. The vectors pBTM116, pACTII, pACTII-GAL4<sub>AD</sub>:CDK4, pBTM116-p16L65P:lexABD, and pBTM116-p16WT:lexABD harbour the genes *lexABD*, *GAL4AD*, *GAL4AD:CDK4*, *p16L65P:lexABD*, and *p16WT:lexABD*, respectively. Different pairs of vectors were transformed into the yeast strain RAY43 and grown on synthetic complete media lacking leucine and tryptophan. The listed values represent the mean of either at least 28 separate assays performed at 30°C or 20 separate assays performed at 37°C. Statistically significant differences ( $\alpha=0.05$ ) were observed between the wild-type variant and the L65P mutant at both 30°C ( $p=0.002$ ) and at 37°C ( $p=0.009$ ).



**Figure 4** Parametric multipoint linkage analysis of chromosomes 1. The analysis was performed using GENEHUNTER (v. 2.0  $\beta$ ), assuming an autosomal dominant inheritance model with 50% penetrance. Forty seven families were tested with 31 dinucleotide microsatellite markers at approximately 10 cM. No evidence for linkage at any locus is shown.



**Figure 5** Parametric multipoint linkage analysis of chromosome 9. The analysis was performed using GENEHUNTER (V. 2.0  $\beta$ ), assuming an autosomal dominant inheritance model with 50% penetrance. Forty six families were tested with 20 dinucleotide microsatellite markers at approximately 10 cM. No evidence for linkage at any locus is shown.

kindreds overall, but only 7.7% (1/13) in families with  $\geq 3$  affected individuals. If we restrict the assessment to only the families of the Emilia Romagna and Marche regions, 6.8% of families were positive for any mutation, and this rate is probably representative of the melanoma kindreds of the area, since at least 85% of their melanoma cases were diagnosed at the Bufalini Hospital during our study period.

A recent publication from the Melanoma Genetics Consortium has provided evidence for a novel susceptibility gene for melanoma within chromosome band *1p22*.<sup>6</sup> The study was restricted to melanoma pedigrees comprising at least three affected members, which were mutation negative for both *CDKN2A* and *CDK4*, had no evidence of haplotype sharing in the *9p21–22* region, and were from different areas of the world. We examined this region, as well as *1p36*<sup>7</sup> in our families using 31 microsatellite markers on chromosome 1 and found no evidence for linkage to either 1p region (fig 4). In addition, our families showed no evidence for linkage to chromosome 9 (fig 5). Although we did not use many markers in the *9p21–22* region, there was no suggestion for linkage to chromosome 9p. Further analysis of the remaining chromosomes may reveal other loci possibly linked to melanoma in this Mediterranean population.

It is possible that the clustering of cutaneous malignant melanoma cases in the Emilia Romagna population occurred by chance alone, as the majority of these families have only two relatives with cutaneous malignant melanoma, and a few melanoma cases also carried other tumours (table 1), possibly representing different syndromes, with low probability of *CDKN2A* mutations.<sup>66</sup> In addition, the population of

the area, known for its sea resorts, is likely to have intense exposure to the sun. Notably, two families whose members had a *CDKN2A* mutation and developed melanoma also spent some years in Africa. Previous studies have shown that sun exposure may increase melanoma risk beyond that accounted for by germline mutations alone,<sup>59 62 67</sup> and possibly modify the penetrance of *CDKN2A* gene mutations.<sup>15</sup> However, in Mediterranean countries, and in particular in Italy, incidence rates of melanoma are relatively low, with 4.6 cases/100 000 person years for men and 5.5 cases/100 000 person years for women<sup>68</sup> and a cumulative risk of developing melanoma over a life time of 0.5%.<sup>2</sup> Because of this low incidence, it is unlikely that familial aggregation is due to chance alone in this population. Moreover, all previous studies conducted in Italy on familial melanoma, which identified many individuals with *CDKN2A* mutations (in 33% of all previously screened families), were based on similar numbers of cases per family.<sup>17 19 47</sup> In addition, several of the Romagna kindreds included members with multiple primary melanomas (even in the families with melanomas only in second or higher degree relatives), or melanomas diagnosed at young ages, which often indicates an underlying genetic predisposition to the disease,<sup>50</sup> and three families also had family members with pancreatic cancer, which has been associated with presence of *CDKN2A* mutations.<sup>18 58 69 70</sup> In addition, we found a high frequency of reported breast cancer, as previously noted in families prone to melanoma,<sup>18 69</sup> and of gastric cancer, which is the fourth most common cancer in Europe,<sup>71</sup> but we found no mutations in the *CDH1* and *BRCA2* genes, making it unlikely that these

kindreds represent clustering of different cancer types or syndromes.

Based on these considerations, it is curious that our mutation detection rate was so low. In this Italian sub-population, there exist either non-coding susceptibility alleles of *CDKN2A* that we cannot detect using current methods, or germline alterations of a genes other than *CDKN2A* that play an important role in melanoma predisposition. Therefore, the Emilia Romagna population may prove to be a rich source for further genetic studies in melanoma in the future.

Among the four *CDKN2A* mutations identified in our study, one was not reported before: a leucine to proline missense mutation at position 65. Perturbations in the primary structure of p16 can lead to profound loss of its inhibitory activity,<sup>72</sup> but existing functional assays have frequently reached conflicting conclusions.<sup>73</sup> We studied the possible effects of the mutation on both the predicted protein structure (fig 2) and its experimentally determined interaction with CDK4 (fig 3). The leucine at position 65 is not conserved across species, and molecular modelling suggests that its replacement by proline will produce surprisingly little effect on the tertiary structure of the protein. In keeping with this supposition, the yeast two hybrid interaction assay demonstrated only a modest reduction in the binding of the L65P mutant to CDK4, when compared with the wild-type p16. Nonetheless, the corresponding allele does cosegregate with disease in at least one family. The phenotypic effect of L65P mutation may exceed that predicted by the yeast assay. This observation is in keeping with that of other investigators. For instance, Becker et al<sup>74</sup> have demonstrated that some p16 mutants fail to induce growth arrest despite retaining normal binding to CDK4. They suggested that such p16 mutations may confer a predisposition to melanoma through a mechanism not yet identified. We speculate that p16 interacts with as yet unidentified proteins, and thus plays a role in tumour suppression that is distinct from its effect on the retinoblastoma pathway, mediated by CDK4.

In addition, another protein may be defective and modify the effect of the mutant L65P in this family. The *melanocortin-1 receptor* (*MC1R*) gene (OMIM 155555) on chromosome 16q24.3 is a possible low-penetrance candidate gene which has been associated with fair complexion and melanoma in a few families,<sup>75</sup> and may act as a modifier of the *CDKN2A* gene.<sup>76-77</sup> We screened the 11 members of the family carrying the p16 L65P mutation for the most common variants of the *MC1R* gene, as part of an on-going study on the association between *MC1R* variants and melanoma. Nine individuals (81%) carried *MC1R* variants, and precisely the missense V92M and silent T314T. All subjects with p16 L65P, including the three living melanoma cases and the unaffected individual, carried these variants. Thus, *MC1R* does not appear to be informative in this family.

Many melanoma cases identified in our study, as well as in a parallel case-control study of sporadic melanoma conducted in the same area,<sup>22</sup> were advanced in stage. This suggests that this population does not generally appreciate the risk for melanoma, and would benefit from public health campaigns against unprotected sun exposure, and from frequent screenings and better understanding of the etiology of the disease. As the incidence and mortality of melanoma continue to increase, particularly in Southern European countries such as Italy, identification of novel genes may have considerable clinical impact on familial melanoma.

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